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On May 9, 2015 we began an approved 1-year no-cost extension in order to complete the project.

14. ABSTRACT
In this Year 4 report we integrate our major results to date to show the overall advances that we have made in the optimization of a small volume therapy that increases survivability of lethal hemorrhagic shock in rats. This includes our results using a small-volume (1 ml/kg) resuscitation fluid based on hibernation physiology that has three main components: 4 M D-stereoisomer of beta-hydroxybutyrate (BHB), 43 mM melatonin, and 20% DMSO. Ten-day mean survival showed a dose-dependent trend: rats survived longer with higher concentration of infused BHB (4 M BHB, 7.38 ± 1.75 days; 2 M BHB, 5.25 ± 2.22 days; 0.4 M BHB, 2.07 ± 2.05 days). Administering 4 M BHB without melatonin resulted in low mean survival times (4.38 ± 1.42 days). All treatments containing both 4 M BHB and melatonin, regardless of melatonin concentration, resulted in mean survival times of ~7.5 days. **Conclusions:** There is a dose-dependent trend in which higher BHB concentration resulted in higher percent survival over 10 days. Melatonin provides therapeutic effects at very low concentrations evident by survival when administering a solution containing 10^6-fold lower melatonin than previously published. Melatonin is essential for survival since 4 M BHB without melatonin had a considerably reduced survival rate.

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Introduction

We previously published a small-volume (1 ml/kg) resuscitation fluid based on hibernation physiology that has three main components: 4 M D-stereoisomer of beta-hydroxybutyrate (BHB), 43 mM melatonin, and 20% DMSO (13). Only one concentration of each component were originally tested. In this study we worked towards optimization of this fluid to enhance survival in a rat model of hemorrhagic shock.

Approach: Two separate dose-ranging studies were conducted for BHB and melatonin in animals with 60% blood loss. BHB was administered at either 4 M, 2 M, or 0.4 M concentration in conjunction with 4.3 mM melatonin and 10% DMSO. Subsequently, melatonin was administered at either 4.3 mM, 0.43 mM, 0.0043 mM, 0.000043 mM, or 0 mM concentration in conjunction with 4 M BHB and 2% DMSO.

Results: Ten-day mean survival showed a dose-dependent trend: rats survived longer with higher concentration of infused BHB (4 M BHB, 7.38 ± 1.75 days; 2 M BHB, 5.25 ± 2.22 days; 0.4 M BHB, 2.07 ± 2.05 days). Administering 4 M BHB without melatonin resulted in low mean survival times (4.38 ± 1.42 days). All treatments containing both 4 M BHB and melatonin, regardless of melatonin concentration, resulted in mean survival times of ~7.5 days.

Conclusions: There is a dose-dependent trend in which higher BHB concentration resulted in higher percent survival over 10 days. Melatonin provides therapeutic effects at very low concentrations evident by survival when administering a solution containing 10^6-fold lower melatonin than previously published. Melatonin is essential for survival since 4 M BHB without melatonin had a considerably reduced survival rate.

Body

Every person in the world is at risk of trauma regardless of social standing, race, religion, or political ideology. Five million people have trauma-related deaths worldwide every year. In the US, traumatic injury is the main cause of death amongst individuals ages 1 to 44. In the year 2000, trauma cost $117 billion on medical care; 10% of the total US medical expenses (1).

Hemorrhage is a problem of major importance in traumatic injury. Hemorrhagic shock is the leading cause of death and complications after trauma, both military and civilian (2). It is also the number one cause of preventable deaths since trauma casualties often occur due to an inability for victims to access medical facilities in a timely manner (3).The effective control of hemorrhage and the development of more efficient resuscitation strategies can save lives (4).

Natural hibernation in mammals is not accompanied by a massive loss of blood due to hemorrhage; however, it exemplifies an exceptional physiological state characterized by a reduction in cardiac output and blood pressure comparable in magnitude to hemorrhagic shock. Furthermore, classic hibernation patterns include multiple days of greatly reduced blood flow resulting from heart beat reduction to 3-10 bpm when animals are in torpor. These hypothermic torpor bouts are regularly interrupted with brief periods of normothermia (37°C) and normal heart rates of 300-400 bpm (5). This dramatic change in animal physiology resembles ischemia/reperfusion events seen in non-hibernating mammals, however the brain and other tissues of hibernators are protected from the pathology of ischemia (6) and reperfusion injury (7). Such protection is achieved by the employment of an array of inherent adaptations present in the hibernating animal (8).

Hypertonic resuscitation was first explored in the 1960s but it was not until the 1980s when interest in this resuscitative strategy germinated. In fact, the term “small-volume resuscitation” was not common until 1984 (9), and the first human experiments were not conducted until 1987 (10). Despite the use of small-volume resuscitation fluids in both experimental and clinical settings (11), they are still not widely employed. Furthermore, clinical approaches for resuscitation are in continuous re-assessment because there is no general agreement in the clinical setting as to which fluid therapy provides the most benefit (12).

In 2010 our laboratory developed a small-volume resuscitation fluid based on hibernation physiology composed of the D-stereoisomer of β-hydroxybutyrate (BHB) and melatonin referred to as BHB/M (13). BHB/M was developed in a rat model of massive blood loss with the goal of providing a portable fluid that would expand the window of opportunity (aka "golden hour") for transport to medical facilities and long-term survival after blood return. We found after 1 hour of 60% blood loss, 4% fluid replacement with BHB/M significantly prolonged survival up to ten days post-blood return compared to control solutions (13). The same solution has been tested in a porcine model of hemorrhagic shock where a survival benefit was also observed (14). However, in both the small and large animal study, only one concentration of each of the components of BHB/M was tested – 4 M BHB, 43 mM melatonin.

BHB/M is highly portable as it is administered in very small volumes (1 ml/kg) and has the potential for self-administration. This is of particularly importance in the military setting where trauma resulting in blood loss can occur in remote locations. In the Korean War, evacuation time of the severely wounded was 3 hours; in the Vietnam War, 83 minutes (15). These times are still outside of the scope of the current "golden hour" for hemorrhagic shock. For that reason, there is still a need for the development of a resuscitation strategy that will allow injured soldiers to survive long enough to gain access to proper medical care.

The objective of the work presented here is to enhance survival in a rat model of hemorrhagic shock by optimizing the composition and delivery of BHB/M.

Key Research Accomplishments

Comparison of two different therapy delivery protocols

Two groups of animals were treated identically except one group received a continuous infusion of therapeutic solution beginning after 60% blood loss. No statistical differences (*p*>0.05*)* were observed in 24-hour survival when comparing the single Bolus Only (mean survival 496.67 ± 314.59 min. n=6) to the single Bolus followed by Slow Infusion (mean survival 149.20 ±142.71 min. n=5) protocol (Figure 3). Based on these results, subsequent studies designed to optimize composition used a single bolus only. All sham-operated animals lived until the experimental end point of 10 days.

Optimization of Therapy Composition

The BHB/M therapy reported previously was developed for hemorrhagic shock treatment and its composition was based largely on animal observations. The objective here was to optimize the composition based on empirical evidence.

Melatonin Dose-Ranging Study I

The formulation of BHB/M containing 4 M BHB with 43 mM melatonin in 20% DMSO (n=6) was compared to a solution containing 4 M BHB, 4.3 mM melatonin and 10% DMSO (n=6). Survival curves (Figure 4) were compared 24 hours and 10 days after 60% blood loss. 24-hour survival showed no statistical differences (*p*>0.05*)* between BHB/M (mean survival 21.0 ± 2.74 hrs) and 4 M BHB, 4.3 mM melatonin and 10% DMSO (mean survival 21.0 ± 2.74 hrs). There were also no statistical differences (*p*>0.05*)* in survival at 10 days (6.4 ± 2.0 days and 7.4 ± 1.8 days, respectively; Table 1).

BHB Dose-Ranging Study

BHB concentrations of 4 M (n=6), 2 M (n=5) and 0.4 M (n=5) were compared 24 hours and 10 days after 60% blood loss (Figure 5). Table 2 summarizes pairwise comparisons results. In short, at 24 hours, the 0.4 M BHB treatment had statistically lower survival (*p*<0.05) than the 4 M BHB and the 2 M BHB treatments. At 10 days, only the difference between 0.4 M and 4 M BHB was upheld (*p*<0.05). However, 10-day mean survival showed a dose-dependent

trend where the higher concentrations of BHB resulted in longer survival (4 M BHB, 7.4 ± 1.8 days; 2 M BHB, 5.3 ± 2.2 days; 0.4 M BHB, 2.1 ± 2.1 days).

Melatonin Dose-Ranging Study II

Survival curves for the concentrations of melatonin shown in Figure 6 were compared 24 hours and 10 days after 60% blood loss. Pairwise comparisons are summarized in Table 3. No treatment differences were observed at either 24 hours or 10 days after 60% blood loss. However, at 10 days, only the treatments with 0 mM melatonin (4.4 ± 1.4 days), 0.0043 mM Mel (6.6 ± 1.6 days), and the NaCl control (4.6 ± 1.4 days) were different ($p<0.05$) from the sham group.

Reportable Outcomes

Comparison of two different therapy delivery protocols

Two groups of animals were treated identically except one group received a continuous infusion of therapeutic solution beginning after 60% blood loss. No statistical differences ($p>0.05$) were observed in 24-hour survival when comparing the single Bolus Only (mean survival 496.67 ± 314.59 min. n=6) to the single Bolus followed by Slow Infusion (mean survival 149.20 ±142.71 min. n=5) protocol (Figure 3). Based on these results, subsequent studies designed to optimize composition used a single bolus only. All sham-operated animals lived until the experimental end point of 10 days.

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Physiological Constants

BHB Dose-Ranging Study

MAP was higher in shams at T_{10}, T_{20}, and T_{30} ($p<0.05$) compared to all hemorrhaged groups. This is to be expected as shams only had blood drawn for sampling. At T_{90}, all hemorrhaged groups were 20-30 mmHg lower than sham-operated animals. However only the published formulation of BHB/M, the treatment with 4 M BHB, and the treatment with 0.4 M BHB showed statistical differences ($p<0.05$) when compared to shams. Sham animals had higher HR than all other treatments at T_{10} ($p<0.05$), T_{20} ($p<0.05$), and T_{30} ($p<0.05$). By T_{90}, hemorrhaged animals had a HR close to that of shams. All animals presented a gradual decrease in rectal temperature as a result of anesthesia. However, the reduction in hemorrhaged animals was steeper than in shams.

Melatonin Dose-Ranging Study II

MAP was lower in all treatments compared to shams ($p<0.05$) through the blood withdrawal phase. During the one-hour hemorrhage period MAP rose steadily to levels close to those of sham animals. After a blood transfusion, MAP reached levels higher than baseline. Sham-operated animals had higher HR than all hemorrhaged groups through the experiment, with T_0 being the exception. All groups, including shams, had gradual decreases in rectal temperature throughout the experimental protocol. However, sham-operated animals consistently showed

rectal temperatures a few degrees above all hemorrhaged animals. Sham-operated animals were excluded when comparing animals that survived to 10 days after 60% blood loss and those that did not because the shams were not hemorrhaged and all lived to 10 days, potentially skewing the results. MAP, HR, and rectal temperature were higher at T_{90} in animals that lived to 10 days (p<0.05). HR was also higher at T_{105} (p<0.05) in 10-day survivors.

Sham rats were excluded from Cox Proportional Hazards regression analyses also. At T_{90}, lower MAP (p<0.05; HR=0.97), HR (p<0.05; HR=0.99), and rectal temperature (p<0.05; HR=.49) resulted in lower survival. Higher rectal temperature (p<0.05; HR=2.29) at T20 lead to a decrease in survival.

Whole-Blood Parameters

BHB Dose-Ranging Study

Treatments differences in pH were observed at T_0. This is more reflective of individual variation since all animals have been instrumented as per the protocol in the methods section and no infusion has taken place at this time point. All groups presented reductions in pH, this could be in part as a result in rectal temperature drops, even though results were temperature-corrected. The 4 M BHB and 2 M BHB treatments had lower pH than shams at T_{30} (p<0.05). However, the differences were only 0.04 and 0.15 pH units, respectively.

Statistical differences in tHb were not observed until T_{30} between sham-operated animals and all hemorrhaged groups (p<0.05) as the loss of blood is accompanied by a reduction in red cell mass (15). A blood transfusion restored tHb levels in close to baseline in hypovolemic animals. sO_2 at T_0 was slightly decreased in all groups, probably as a result of anesthesia as isoflurane has been known to cause dips in sO_2 at concentrations larger than 2% (17). pO_2 was higher in all hemorrhaged treatments compared to shams at T_{10} (p<0.05), T_{20} (p<0.05), T_{30} (p<0.05), T_{90} (p<0.05), and T_{105} (p<0.05). This is contrary to the expectation that pO_2 will be depressed in shocked animals (15). However, it reflects intact respiratory function. Changes in pCO2, though minimal and not statistically different between treatments (p>0.05), seemed to parallel changes in blood pressure.

Hyperkalemia is expected to occur in sever shock (15). In rats subjected to hypovolemia, circulating K^+ levels increased as the hemorrhagic phase progressed but returned to levels close to baseline during the shock period. In contrast, blood K+ in shams was lower from T0 to T10 but continually increased, resulting in statistically higher levels (p<0.05) when compared to all hemorrhaged groups at the end of the surgical procedure. However, mean K^+ concentrations were not outside of the normal range (3.9-9.2 mmol/L) (18) at any time point in any group. Hemorrhage lowers blood Na^+ levels (15). Since the BHB we used comes in its sodium salt form, circulating Na^+ increased upon infusion. The one-hour shock period allowed Na^+ to go back to homeostatic values. In shams, blood Na^+ increased after the first few blood samples and then slowly returned to its basal range. Ca^{++} blood concentrations varied between groups enough to show statistical differences at T_0 (p<0.05). However, this is just reflective of individual variation between the animals assigned to each group and the Ca^{++} levels were not outside of the reference values (18). With blood withdrawal, circulating Ca^{++} increased, but then decreased upon infusion. In fact, Ca^{++} levels somewhat mirrored the curves for Na^+ and MAP. Nonetheless, these fluctuations remained within the normal value range. Circulating Cl^- levels fluctuated without a specific pattern or trend but tended towards the higher end of the reference levels. Sham-operated animals had lower Glu and Lac levels than all other treatments (p<0.05) at all time points except T_0, consistent with a normal response to shock (15).

Melatonin Dose-Ranging Study II

All groups were indistinguishable at T_0. tHb was consistently higher in shams compared to hemorrhaged animals. Those differences were no longer present after a blood transfusion. pO_2 was consistently lower in shams compared to all other treatments except at T_0 and T_{105}. The opposite was observed with pCO_2. K^+ levels in the hemorrhaged groups seemed to mirror MAP. In the sham group, circulating K^+ decreased initially and then gradually increased.

In hemorrhaged animals, blood Na+ decreased with blood withdrawals and increased with treatment infusion. Over the one-hour shock period, circulating Na+ increased slightly. In sham animals, Na^+ levels stayed relatively constant. Blood Ca^{++} concentrations varied and seemed to somewhat mirror MAP. However, those changes never fluctuated beyond the reference values

for Ca^{++}. Cl^- was higher in the animals infused with the NaCl control treatment from T_{20} onwards. Both Glu and Lac remained at basal levels in sham-operated animals while they constantly increased in hemorrhaged rats.

Sham rats were not included when comparing animals that lived to 10 days after 60% blood loss and those that died. All sham-operated animals survived to 10 days since they were not hemorrhaged and including them in this analyses could misrepresent the data. At T_0, sO_2 was higher in non-survivors (p<0.05). tHb was lower in 10-day survivors at T_{10} (p<0.05). Blood Lac was higher at T_{20} in animals that did not live to 10 days (p<0.05). At T_{90}, 10-day survivors had higher pH (p<0.05) and pCO_2 (p<0.05); non-survivors had higher sO_2 (p<0.05), K^+ (p<0.05), and Lac (p<0.05). At T_{105}, pH was higher in rats that lived to 10 days (p<0.05); animals that died before the 10-day end point had higher circulating Lac (p<0.05).

Sham-operated animals were also excluded from regression analyses. At T_0, higher sO_2 (p<0.05; HR=1.22) resulted in lower survival. At T_{10}, increased pH (p<0.05; HR=301.33) and increased tHb (p<0.05; HR=4.56) decreased survival. Reduced tHb at T_{20} (p<0.05; HR=0.16) reduced survival. Lower pCO_2 (p<0.05; HR=0.85) and higher K+ (p<0.05) at T_{90} caused a reduction in survival. At T_{105}, lower pH (p<0.05; HR=0.05) and higher Lac (p<0.05; HR=1.03) resulted in lower survival.

Histopathological Scoring

Histopathological analyses are important for our research because in trauma, death is mostly encountered at three points: 1) within the first hour, 2) within the next 24 hours, 3) after days or weeks (19). Since out post-operatory monitoring is not comprised of weeks, observing micro anatomical changes in different tissues provide information regarding the health status of the experimental subjects that survived the entire 10 days. It also helped us identify whether those animals would have kept living indefinitely or would have been likely to suffer health consequences in the near future. Histological scoring was only conducted on the second melatonin dose-ranging study. Ten-day survivors administered 0.000043 mM melatonin had lower histopathological scores for intestine compared to those infused with 4.3 mM melatonin (p<0.05). However, the average score for this treatment represented only moderate damage,

suggesting that even though there was a stronger inflammatory response in this group, ten days after 60% blood loss the inflammation is being resolved.

Plasma Parameters

BHB Dose-Ranging Study

Plasma BHB levels were higher in animals infused with 4 M BHB compared to those administered 0.4 M BHB at T_{20} ($p<0.05$) and T_{30} ($p<0.05$). These differences were expected because they were consistent with the different concentrations infused. At T_{105}, the concentration of BHB in the plasma was higher in animals that died between day 0 and day 9 compared to those that survived until the 10 day end point ($p<0.05$). Lower BHB levels at T_{20} ($p<0.05$) and circulating BHB at T_{105} ($p<0.05$) resulted in decreased survival.

Melatonin Dose-Ranging Study II

Circulating TNF-α levels were determined for a representative sample (n=3 per treatment) of animals from the sham group as well as animals administered 4.3 mM melatonin, 0.000043 mM melatonin, 0 mM melatonin, and the NaCl control for T_{20}, T_{30}, and T_{90}. However, when shams and NaCl controls were not considered, a dose dependent trend was observed at T_{30} where the higher the melatonin administered, the lower the circulating TNF-α. Also at T_{30}, TNF-α plasma levels were lower in animals that lived until the end of the experiment compared to those that died prematurely ($p<0.05$). TNF-α did not seem influence survival.

Conclusion

There are three potential mechanisms by which BHB/M improves survival of hemorrhagic shock and they have immediate, short-term, and long-term effects. The immediate effects are unspecific and can be attributed to its osmolarity. It is well known that when administering a hypertonic solution into a blood vessel the changes in solute concentration will drive intracellular water into the intravascular space, expanding the plasma volume (20). This expansion can support an increase in MAP allowing blood to continue to circulate and minimizing ischemia (11).

In the short term, BHB/M action is twofold. First, BHB acts as fuel source preserving cell function. One of the main outcomes of hemorrhagic shock is adenosine triphosphate (ATP) depletion which leads to cell death (20). BHB dehydrogenase converts BHB into acetoacetate which binds CoA transferred from succinyl-CoA via succinyl-CoA transferase (SCOT). Acetoacetyl-CoA is subsequently converted into two acetyl-CoA molecules that can maintain ATP production via the TCA cycle. Hence, a single molecule of BHB is as energy efficient as a molecule of glucose without the potential of generating lactate. SCOT is the rate-limiting enzyme in this process and is also up-regulated in the heart of hibernating ground squirrels (21) during periods of torpor and low blood flow when BHB levels are elevated and glucose levels are depressed (22).

Second, melatonin is a powerful antioxidant and free radical scavenger. Reperfusion of an ischemic cell generates reactive oxygen species (ROS) which can damage cell membranes, directly injure DNA and proteins, and exacerbate inflammatory processes (23, 24), all of which lead to apoptosis. Melatonin can neutralize ROS, minimizing reperfusion injury. Furthermore, the products of its reaction with free radicals, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), also possess antioxidant properties (25). The long term effects of BHB/M can also be attributed to melatonin as an immunomodulator, primarily through the scavenging of ROS and the inhibition of the activation of nuclear factor kappa B (NF-κB) (26). Limiting the inflammatory response in hemorrhagic shock and ischemia/reperfusion injury is paramount for long term survival. Patients who initially survive a

hemorrhagic event could die weeks after the initial injury as the result of multiple organ failure (MOF) - a disproportionate self-destructive inflammation leading to the malfunction of organs not involved in the original traumatic event (19).

So What?

Our results show that a single 1 ml/kg bolus of BHB/M provided the same survival benefit as a bolus plus a slow infusion. In fact, animals that were administered a single bolus lived longer than those in which a slow infusion was continued. The lack of statistical difference could be the result of a small sample size. It is possible that a single bolus allows the organism to balance the osmolarity and electrolyte content of the blood. Continuing to infuse a hypertonic solution may exceed the individual's ability to compensate for the osmolar load and electrolyte imbalance (11).

We demonstrated that it is important to maintain the BHB concentration at 4M in order to obtain maximum survival. The sodium salt of BHB is responsible for both the fluid shift from the intracellular space into the intravascular space and as a carbon source that maintains ATP production. Consequently, 2 M BHB would account result in only half the plasma expansion and half the fuel source. Reducing the concentration by ten-fold would not even manage to increase plasma BHB above the baseline circulating levels observed in our study and by Klein *et al* (13). Administering 4 M NaCl could not support survival as effectively as 4 M BHB because NaCl only has a circulatory benefit but does not fuel the production of ATP which is essential for the maintenance of normal cellular function.

Melatonin can be administered at concentrations a million-fold lower than the previously published formulation (13) and still support survival. This is reasonable since serum melatonin peaks in rats are ~8.61×10^{-7} mM (27). Hence, administering a 1 ml/Kg bolus of 43 mM melatonin, the way it occurred in the experiments by Klein *et al* (13), would result in plasma levels almost fifty thousand times higher than peak. When 4 M BHB was administered without melatonin, initial survival was observed, but it could not be supported long term. This supports our assumption that long term survival is achieved through mechanisms involving the immunomodulatory properties of melatonin.

The anti-inflammatory properties of melatonin may be dose-dependent. Our histological results showed that the lowest dose of melatonin administered, 0.000043 mM, had statistically higher injury scores in small intestine than the one administered the highest dose of 4.3 mM in our second melatonin dose-ranging study. It is possible that other dose-dependent differences were masked by statistic variability and low sample size. Furthermore, the melatonin response observed at T_{30} in TNF-α levels also support the idea of a dose-dependent anti-inflammatory action of melatonin. However, these differences do not seem to affect survival, supporting a reduction in the concentration of melatonin in the composition of BHB/M in a rat model.

In summary, our experiments support an adjustment in the composition of the previously published BHB/M. The ketone component of BHB/M, beta-hydroxybutyrate, should remain at a concentration of 4 M. Melatonin can be administered at a concentration 10^6-fold lower than the previously published concentration (13) without affecting survival rate. Adjusting melatonin to a lower level has the benefit of reducing the concentration of the solvent DMSO. This is advantageous since there is some controversy over the use of DMSO (28). We also demonstrated that a slow infusion after a bolus administration is not necessary. This is a highly desirable trait as it increases the feasibility for self-administration in combat scenarios.

References

1. Kauvar DS, Wade CE. The epidemiology and modern management of traumatic hemorrhage: US and international perspectives. Critical Care. 2005;9(Suppl 5):S1-S9.

2. Rhee P, Alam H, Ling G. Hemorrhagic Shock and Resuscitation: Trauma Research at the Trauma Research and Readiness Institute for Sugery. In: Tsokos G, Atkins J, editors. Combat Medicine: Basic and Clinical Research in Military, Trauma, and Emergency Medicine. Totowa, New Hersey: Humana Press Inc.; 2003.

3. Support ACoSCoTSoATL. Advanced Trauma Life Support Course for Physicians: The Committee; 1989.

4. Alam HB, Koustova E, Rhee P. Combat Casualty Care Research: From Bench to the Battlefield. World Journal of Surgery. 2005;29(0):S7-S11.

5. Geiser F, Mzilikazi N. Does torpor of elephant shrews differ from that of other heterothermic mammals? Journal of Mammalogy. 2011;92(2):452-9.

6. D'Alecy L. Beta-hydroxybutyrate and response to hypoxia in the ground squirrel, Spermophilus tridecemlineatus. Comparative Biochemistry and Physiology B. 1990;96(1):189-93.

7. Andrews MT. Advances in molecular biology of hibernation in mammals. Bioessays. 2007;29(5):431-40.

8. Graf R, Schaller B. "Natural" Tolerance in Hibernators: Can We Learn from Physiological Preconditioning Against Ischemic or Hypoxic Brain Injury? In: Schaller B, editor. Cerebral Ischemic Tolerance: From Animal Models to Clinical Relevance. Hauppauge, New York: Nova Science Publishers, Inc.; 2004.

9. Nakayama S-i, Sibley L, Gunther R, Holcroft J, Kramer G. Small-volume resuscitation with hypertonic saline (2,400 mOsm/liter) during hemorrhagic shock. Circulatory shock. 1984;13(2):149.

10. Holcroft JW, Vassar MJ, Turner JE, Derlet RW, Kramer GC. 3% NaCl and 7.5% NaCl/dextran 70 in the resuscitation of severely injured patients. Annals of surgery. 1987;206(3):279.

11. Kreimeier U, Messmer K. Small-volume resuscitation: from experimental evidence to clinical routine. Advantages and disadvantages of hypertonic solutions. Acta anaesthesiologica scandinavica. 2002;46(6):625-38.

12. Angele MK, Schneider CP, Chaudry IH. Bench-to-bedside review: latest results in hemorrhagic shock. Critical Care. 2008;12(4):218.

13. Klein AH, Wendroth SM, Drewes LR, Andrews MT. Small-Volume d-[beta]-Hydroxybutyrate Solution Infusion Increases Survivability of Lethal Hemorrhagic Shock in Rats. Shock. 2010;34(6):565-72.

14. Mulier KE, Lexcen DR, Luzcek E, Greenberg JJ, Beilman GJ. Treatment with beta-hydroxybutyrate and melatonin is associated with improved survival in a porcine model of hemorrhagic shock. Resuscitation. 2012;83(2):253-8.

15. Carey LC, Lowery BD, Cloutier CT. Hemorrhagic shock. Current problems in surgery. 1971;8(1):1-48.

16. Lee H, Blaufox M. Blood volume in the rat. Journal of Nuclear Medicine. 1985;26(1):72-6.

17. Palahniuk RJ, Shnider SM. Maternal and fetal cardiovascular and acid-base changes during halothane and isoflurane anesthesia in the pregnant ewe. Anesthesiology. 1974;41(5):462-71.

18. Sharp P, Villano JS. The Laboratory Rat: CRC Press LLC; 1998.

19. Baue AE, Faist E, Fry DE. Multiple Organ Failure: Pathophysiology, Prevention, and Therapy: Springer New York; 2000.

20. Kashiwaya Y, Takeshima T, Mori N, Nakashima K, Clarke K, Veech RL. d-β-Hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. Proceedings of the National Academy of Sciences. 2000;97(10):5440-4.

21. Russeth, KP, Higgins, L, and Andrews, MT. Identification of proteins from non-model organisms using mass spectrometry: Application to a hibernating mammal. J. Proteome Res. 2006; 5, 829-839.

22. Andrews, MT, Russeth, KP, Drewes, LR, and Henry, PG Adaptive mechanisms regulate preferred utilization of ketones in the heart and brain of a hibernating mammal during arousal from torpor. 2009; Am. J. Physiol. 296, R383-393.

23. Sanada S, Komuro I, Kitakaze M. Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures. American Journal of Physiology-Heart and Circulatory Physiology. 2011;301(5):H1723-H41.

24. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. The FASEB Journal. 2003;17(10):1195-214.

25. Mayo JC, Sainz RM, Tan D-X, Hardeland R, Leon J, Rodriguez C, et al. Anti-inflammatory actions of melatonin and its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), in macrophages. Journal of neuroimmunology. 2005;165(1):139-49.

26. Cuzzocrea S, Reiter RJ. Pharmacological action of melatonin in shock, inflammation and ischemia/reperfusion injury. European journal of pharmacology. 2001;426(1):1-10.

27. Benot S, Molinero P, Soutto M, Goberna R, Guerrero JM. Circadian variations in the rat serum total antioxidant status: Correlation with melatonin levels. Journal of Pineal Research. 1998;25(1):1-4.

28. Davis PW. An Incipient "Wonder Drug" Movement: DMSO and the Food and Drug Administration. Social Problems. 1984:197-212.

Appendices

Tables

Table 1. Melatonin Dose-Ranging Study I: Mean survival time in animals subjected to 60% blood loss at 24 hours and ten days.

Treatment		Mean ± SD	
		24 Hrs	10 Days
4.3 mM Mel		21.00 ± 2.74	7.375 ± 1.75
43 mM Mel		21.00 ± 2.74	6.375 ± 2.00
Sham		24.00	10.00
Treatment Comparisons		*p*-value	
		24 Hrs	10 Days
4.3 mM Mel	43 mM Mel	1.0000	0.7980
4.3 mM Mel	Sham	0.1760	0.0143
43 mM Mel	Sham	0.1760	0.0143

Mean survival time calculated as the area under the Kaplan-Meier curve. Units are hours for the calculations at 24 hours and days for the calculations at ten days. The treatment labeled as 43 mM Mel contains 4 M BHB with 43 mM melatonin in 20% DMSO (n=6); the treatment labeled as 4.3 mM Mel contains 4 M BHB with 4.3 mM melatonin in 10% DMSO (n=6). Statistically significant *p*-values are colored in red and underlined. Abbreviations: Mel, melatonin.

Table 2. BHB Dose-Ranging Study: Mean survival time in animals subjected to 60% blood loss at 24 hours and ten days.

Treatment		Mean ± SD	
		24 Hrs	10 Days
0.4 M BHB		6.50 ± 4.64	20.07 ± 2.05
2 M BHB		20.40 ± 3.22	5.25 ± 2.21
4 M BHB		21.00 ± 2.74	7.38 ± 1.75
Sham		24.00	10.00
Treatment Comparisons		*p*-value	
		24 Hrs	10 Days
0.4 M BHB	2 M BHB	0.0398	0.0803
0.4 M BHB	4 M BHB	0.0222	0.0472
0.4 M BHB	Sham	0.0004	0.0004
2 M BHB	4 M BHB	0.8920	0.4990
2 M BHB	Sham	0.1380	0.0041
4 M BHB	Sham	0.1760	0.0102

Mean survival time calculated as the area under the Kaplan-Meier curve. Units are hours for the calculations at 24 hours and days for the calculations at ten days. Sample sizes are as follows: 4 M BHB, n=6; 2 M BHB, n=5; 0.4 M BHB, n=5. All solutions contained 4.3 mM melatonin and 10% DMSO. Statistically significant *p*-values are colored in red and underlined. Abbreviations: BHB, D-stereoisomer of beta-hydroxybutyrate.

Table 3. Melatonin Dose-Ranging Study II: Mean survival time in animals subjected to 60% blood loss at 24 hours and ten days.

Treatment	Mean ± SD	
	24 Hrs	10 Days
0 mM Mel	18.6 ± 3.20	4.38 ± 1.42
0.000043 mM Mel	21.71 ± 2.76	7.71 ± 1.10
0.0043 mM Mel	18.60 ± 3.20	3.58 ± 1.60
.43 mM Mel	22.20 ± 2.30	7.63 ± 1.44
4.3 mM Mel	20.40 ± 3.22	7.75 ± 1.49
NaCl Control	18.60 ± 3.20	4.58 ± 1.42
Sham	24	10.00

Treatment Comparisons		*p*-value	
		24 Hrs	10 Days
Sham	0 mM Mel	0.0555	0.0009
Sham	4.3 mM Mel	0.1280	0.0555
Sham	.43 mM Mel	0.2940	0.0555
Sham	0.0043 mM Mel	0.0555	0.0229
Sham	0.000043 mM Mel	0.2940	0.0555
Sham	NaCl Control	0.0555	0.0009
0 mM Mel	4.3 mM Mel	0.6150	0.1200
0 mM Mel	.43 mM Mel	0.2760	0.0792
0 mM Mel	0.0043 mM Mel	1.0000	0.3560
0 mM Mel	0.000043 mM Mel	0.3570	0.0864
0 mM Mel	NaCl Control	1.0000	0.9080
4.3 mM Mel	.43 mM Mel	0.5420	0.9630
4.3 mM Mel	0.0043 mM Mel	0.6150	0.5910
4.3 mM Mel	0.000043 mM Mel	0.6260	0.9630
4.3 mM Mel	NaCl Control	0.6150	0.1310
.43 mM Mel	0.0043 mM Mel	0.2760	0.5630
.43 mM Mel	0.000043 mM Mel	0.9420	1.0000
.43 mM Mel	NaCl Control	0.2760	0.0866
0.0043 mM Mel	0.000043 mM Mel	0.3570	0.6570
0.0043 mM Mel	NaCl Control	1.0000	0.3550
0.000043 mM Mel	NaCl Control	0.3570	0.0866

Mean survival time calculated as the area under the Kaplan-Meier curve. Units are hours for the calculations at 24 hours and days for the calculations at ten days. Sample sizes are as follows: 4.3 mM melatonin, n=10; 0.43 mM melatonin, n=10; 0.0043 mM melatonin, n=10); 0.000043 mM melatonin, n=10; and 0 mM melatonin, n=10. These solutions contained 4 M BHB and 2% DMSO. A control group was also included and were administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=10). Statistically significant *p*-values are colored in red and underlined. Abbreviations: Mel, melatonin.

FIGURE LEGENDS

Figure 1. Optimization of delivery experiments: Experimental timeline. After surgical preparation, animals were hemorrhaged until MAP ~25mmHg. They were then infused with either (A) a single 1 ml/kg bolus, or (B) a bolus followed by a 100 µl/hr slow infusion. In both instances, the bolus infusion was administered within 10 minutes of achieving MAP ~25mmHg. After bolus administration, animals were further hemorrhaged to 60% of their calculated blood volume. No blood was transfused at any time point. Animals were monitored for 24 hours. All 24-hour survivors were euthanized.

Figure 2. Optimization of composition experiments: Experimental timeline. After surgical preparation, animals were hemorrhaged until MAP ~25mmHg and infused with a single 1 ml/kg bolus of solution over a 10 minute period. After bolus administration, animals were further hemorrhaged to 60% of their calculated blood volume and maintained in a shocked state for one hour. One-half (50%) of the shed blood volume was auto-transfused at a rate of 500 µl/min 60 minutes after achieving 60% blood loss. Animals were monitored for 10 days. All animals surviving 10 days were euthanized.

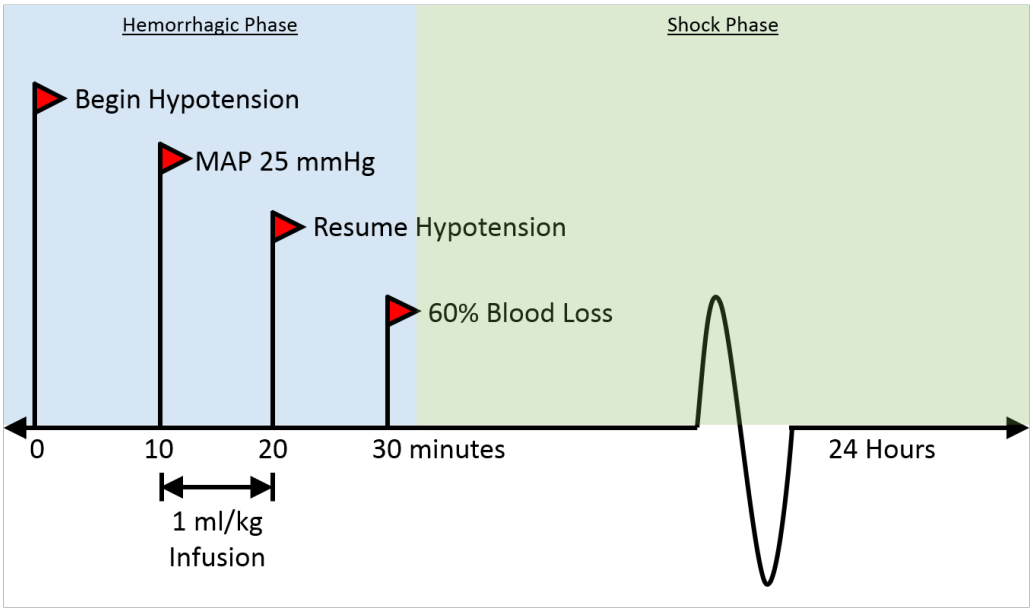
Figure 3. Optimization of delivery experiments: Kaplan-Meier plot of animals subjected to 60% blood loss. Infusion of the published formulation of BHB/M (4 M BHB with 43 mM melatonin in 20% DMSO) was conducted by administering either a single 1 ml/kg bolus (n=6) or a bolus followed by a 100 µl/hr slow infusion (n=5). The groups were not statistically different from each other. Times on the x-axis reflect minutes after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

Figure 4. Melatonin Dose-Ranging Study I: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days. Infusion of either 4 M BHB with 43 mM melatonin in 20% DMSO (n=6) or 4 M BHB with 4.3 mM melatonin in 10% DMSO (n=6) was achieved by administering a single 1 ml/kg bolus. Sham-operated animals are also included in the graph. Times on the x-axis reflect either hours (panel A) or days (panel B) after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

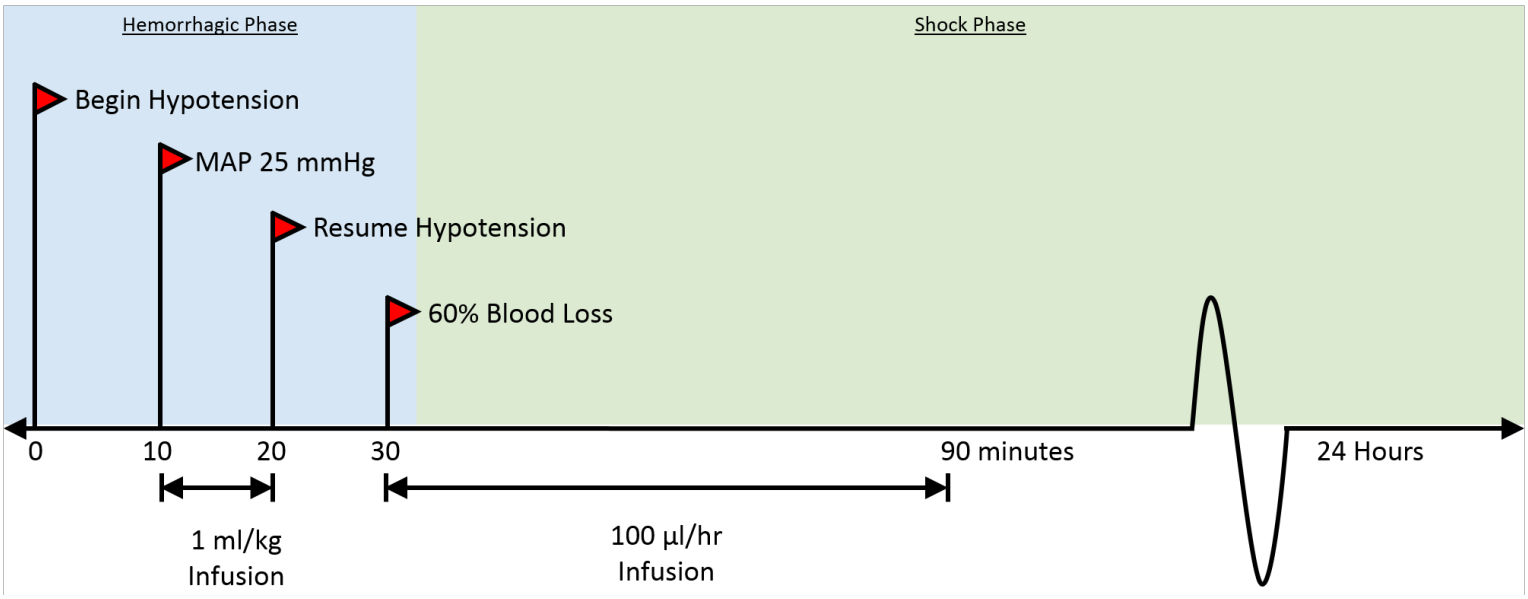
Figure 5. BHB Dose-Ranging Study: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days. Infusion of either 4 M BHB (n=6), 2 M BHB (n=5), or 0.4 M BHB (n=5) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4.3 mM melatonin and 10% DMSO. Sham-operated animals are also included in the graph. Times on the x-axis reflect either hours (A) or days (B) after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

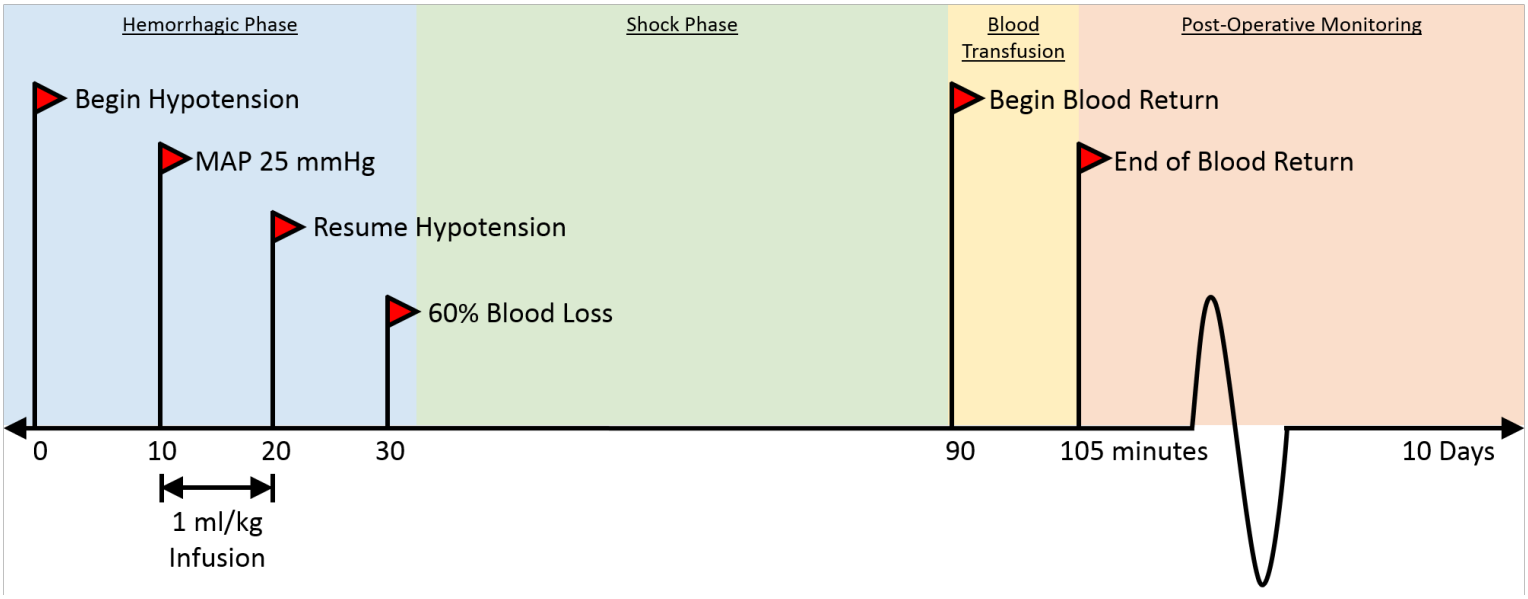
Figure 6. Melatonin Dose-Ranging Study II: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days. Infusion of either 4.3 mM melatonin (n=10), 0.43 mM melatonin (n=10), 0.0043 mM melatonin (n=10), 0.000043 mM melatonin (n=10), or 0 mM melatonin (n=10) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4 M BHB and 2% DMSO. A control group was administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=10). Sham-operated animals are also included in the graph. The time shown on the x-axis reflect either hours (panel A) or days (panel B) after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

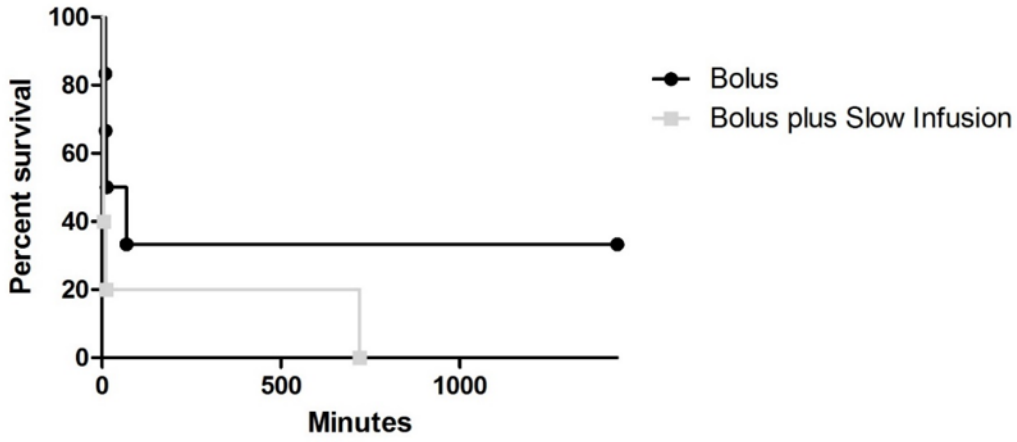
A


Hemorrhagic Phase
Shock Phase
Begin Hypotension
MAP 25 mmHg
Resume Hypotension
60% Blood Loss
0
10
20
30 minutes
24 Hours
1 ml/kg
Infusion

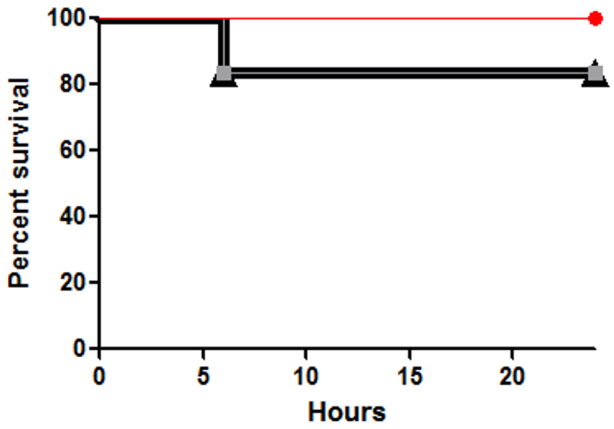
B


Hemorrhagic Phase
Shock Phase
Begin Hypotension
MAP 25 mmHg
Resume Hypotension
60% Blood Loss
0
10
20
30
90 minutes
24 Hours
1 ml/kg
Infusion
100 µl/hr
Infusion

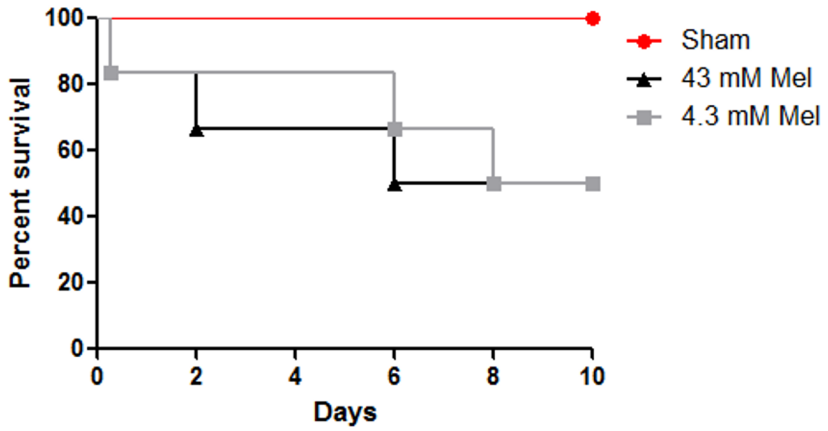

Hemorrhagic Phase
Shock Phase
Blood Transfusion
Post-Operative Monitoring
Begin Hypotension
MAP 25 mmHg
Resume Hypotension
60% Blood Loss
Begin Blood Return
End of Blood Return
0
10
20
30
90
105 minutes
10 Days
1 ml/kg Infusion


100
80
60
40
20
0
Percent survival
0
500
1000
Minutes
Bolus
Bolus plus Slow Infusion

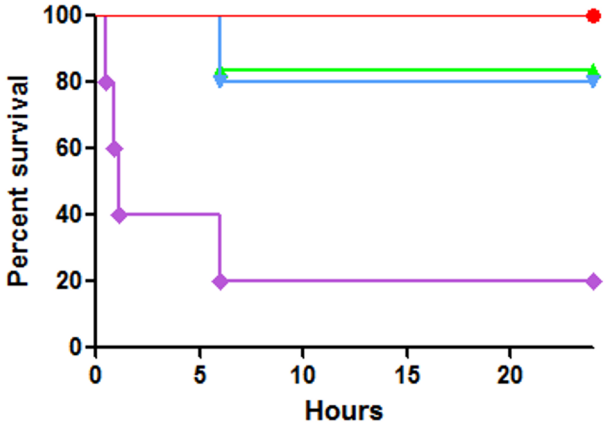
A


Percent survival
100
80
60
40
20
0
0
5
10
15
20
Hours

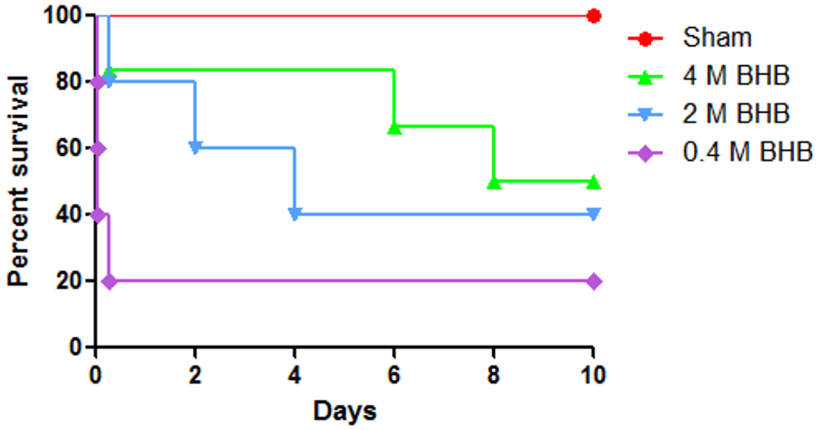
B


Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
Days
Sham
43 mM Mel
4.3 mM Mel

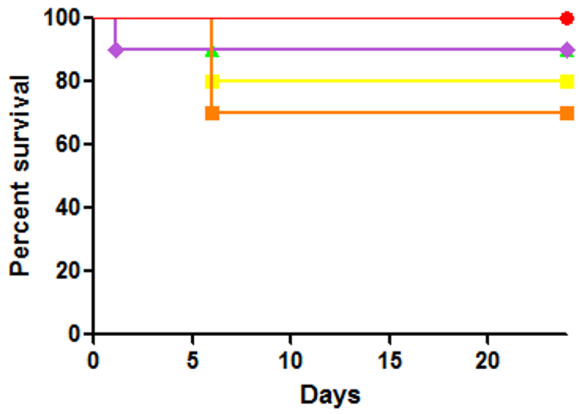
A


Percent survival
100
80
60
40
20
0
0
5
10
15
20
Hours

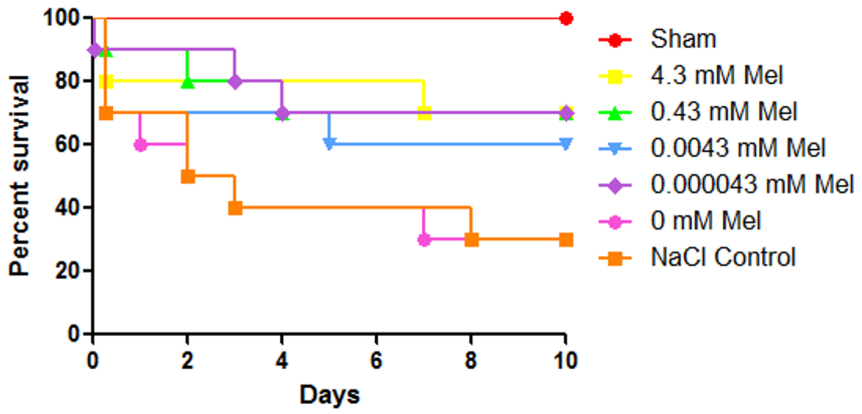
B


Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
Days
Sham
4 M BHB
2 M BHB
0.4 M BHB

A


Percent survival
100
80
60
40
20
0
0
5
10
15
20
Days

B


Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
Days
Sham
4.3 mM Mel
0.43 mM Mel
0.0043 mM Mel
0.000043 mM Mel
0 mM Mel
NaCl Control